Detection of *Enterococcus faecalis* in Necrotic Teeth Root Canals by Culture and Polymerase Chain Reaction Methods

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Objectives: The aim of this study was to investigate the presence of *Enterococcus faecalis* in endodontic infections in both deciduous and permanent teeth by culture and polymerase chain reaction (PCR) methods.

Methods: A total of 145 children aged 5-13 years old were involved in this study. The presence of *E. faecalis* in necrotic deciduous and permanent teeth root canals was studied using culture and polymerase chain reaction methods.

Results: Among 145 molar teeth, 57% (n=83) presented necrotic asymptomatic pulp tissues and were included in this study. Culture and PCR methods detected the test species in 18 and 22 of 83 teeth involved, respectively. *E. faecalis* was cultured from 8 (18%) of 45 necrotic deciduous teeth and from 10 (26%) of 38 necrotic permanent teeth. PCR detection identified the target species in 10 (22%) and 12 (32%) of necrotic deciduous and permanent teeth respectively. Statistically significant difference in the presence of *E. faecalis* in deciduous and permanent teeth was found by culture and PCR methods (P=0.03 and 0.02, respectively). The difference in the presence of *E. faecalis* between two different methods was not statistically significant (P>.05).

Conclusions: The results of the present study confirm that both culture and PCR methods are sensitive to detect *E. faecalis* in root canals. (Eur J Dent 2007;1:216-221)

Key words: *Enterococcus faecalis*; Necrotic tooth; Culture; Polymerase chain reaction.

INTRODUCTION

Enterococci are common inhabitants of the human gastrointestinal and genitor urinary tracts.[1] They are also able to colonize a variety of other sites, including the oral cavity.[2] Enterococci have also been implicated in endodontic infections. Among the enterococci species isolated from root canals, *Enterococcus faecalis* is the most common species. It is a non-fastidious, therapy-resistant microorganism in infected root canals.[3] However; it constitutes a small percentage of the microbial species isolated from root canals of teeth with necrotic dental pulp.[4]

Culture methods have provided a great contribution to, and have still much to offer in

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the elucidation of endodontic diseases. However, molecular approaches to detect and identify microbial species have several advantages when compared with culture. Molecular methods, particularly polymerase chain reaction (PCR), are more specific, accurate, sensitive and rapid than culture, and can detect uncultivable and fastidious microorganisms.[5,6] PCR is a technique, which uses a DNA polymerase enzyme to make a huge number of copies of virtually any given piece of DNA or gene.[7]

Although, culture and molecular techniques have been widely used to detect bacteria in endodontic infections,[8-10] few studies have used them to investigate the microbiota of deciduous teeth.[11-13]

The aim of the present study was to investigate the presence of *E. faecalis* in endodontic infections in both deciduous and permanent teeth by culture and polymerase chain reaction methods.

MATERIALS AND METHODS

A total of 145 children aged 5-13 years old who attended the Ege University School of Dentistry Department of Pedodontics, Izmir, Turkey with an indication for endodontic treatment were involved in this study. All clinical procedures were approved by the Ethical Committee and informed consent was obtained from each parent. A detailed medical and dental history was obtained from each parent. Patients having received antibiotic therapy in the last 3 months or having a systemic disease were not included. Selected teeth showed no significant gingival recession and were free of periodontal pockets > 4 mm deep. Teeth which could not be fully isolated with a rubber dam were also not included in the study.

The following clinical features of each patient were recorded: age, gender, tooth and pulp status, clinical signs and symptoms included history of previous pain, spontaneous pain, tenderness to percussion, pain on palpation, mobility, presence of swelling, wet canal, odor and periapical radiolucency. Among 145 children involved in this study, only the ones with necrotic molar teeth were selected. Endodontic samplings were obtained only from these children.

Microbiological sampling

Eigthy-three (45 deciduous, 38 permanent) endodontic samplings from necrotic molar teeth were obtained during the first visit of root canal therapy. After the tooth crown was cleansed with pumice, a rubber-dam was placed. The tooth and the surrounding field were then cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite solution for 30 s each. The solution was inactivated with 5% sodium thiosulfate.[14,15] Sampling was performed to check the sterility of the operating field before intracanal sampling procedure in all cases.

Access to the root canal was made using sterile burs without water spray. Aseptic techniques were used for instrumentation, during access to and removal of the contents from the pulp space, and sample collection. In each case, a single root canal was sampled in order to confine the microbial evaluation to a single ecologic environment. The criteria used to choose the canal to be microbiologically investigated in the multirooted teeth was the root canal with periapical radiolucency or the largest canal: in the upper molars palatal canal, in the lower molars distal canal.[9,16] Samples were initially collected by means of #15 K-type file with the handle cut off. A sterile #15 file was used to agitate canal contents for 60 s.[10] The file was introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs, and a gentle filing motion was used. Afterwards, two sequential paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in position for 60 s. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal to ensure viable sample acquisition. Chemical active irrigants were never used before sampling.[17] The cut file and two paper points were then transferred to sterile 2 ml eppendorf tubes containing VMGA III transport medium.[18] All samples were processed within 2 hrs. After thoroughly shaking the endodontic sample in a mixer for 60 s (Vortex, Scientific Industries Inc., Springfield, MA), 1 ml of each sample were used for culture and the other 1 ml samples were frozen immediately at -20°C and stored until assayed by PCR.

Microbial isolation and identification

The tubes containing samples in VMGA III were preincubated for 30 minutes (min) at 37°C,

and shaken vigorously in a vortex mixer (Vortex, Scientific Industries, Inc. Springfield, MA) for 60 s. Serial 10-fold dilutions were made up to 1:10^6 in 1% sterile peptone water (Bacto peptone, Difco, Detroit MI, USA). From the serial dilutions, 0.1 ml was transferred and plated on Brucella (BBL, Becton Dickinson Microbiological Systems, Cockeysville, Md) blood agar plates. The plates were incubated in an anaerobic chamber for 48 h and the *E. faecalis* counts were determined as CFU/ml. The purity of the cultures was confirmed by Gram staining, catalase production, colony morphology on blood agar and using a biochemical identification kit (API 20 Strep, bioMerieux: Marcy-l'Etoile, France).

PCR Procedures

Aliquots of each sample (1,0 ml) were centrifuged at 13,000 × g for 10 minutes. The resulting pellets were washed with 500 µL of phosphate-buffered saline, and placed in 200 µL of TE buffer (10mM Tris-Cl pH 7.5, 1 mM EDTA). DNA extraction was performed with "Genomic DNA Purification Kit" (Fermentas GmbH, Germany). Reference DNA from *E. faecalis* (ATCC 29212) was also extracted to serve as positive control. The DNA concentrations in clinical samples and the concentrations of the reference DNA were determined by spectrophotometric measurement of the absorbance at 260 nm. Serial 10-fold dilutions of known concentration of reference DNA of the target species were processed to determine PCR assay sensitivity. The lowest DNA concentration that resulted in a positive PCR product was regarded as indicative of the sensitivity of the assay. Primer specificity was further tested against reference DNA.

PCR primers, with expected amplicon size and thermocycling parameters used in the present study are shown in Table 1.[10,19]

The PCR reaction used to assess the occurrence of all target taxa, was performed in 50 µl of reaction mixture containing 10 µl DNA, 5 µl 10x PCR buffer, 2 mM $MgCl_2$, 1.25 µl Taq DNA polymerase, 0.2 mM dNTP, 1µM specific primer. Negative controls consisting of ultrapure water instead of sample were included with each batch of samples analyzed.[20]

DNA amplification was performed in a thermal cycler Gene Amp®PCR system (Applied Biosystems). Amplicons were stored at –20°C. The amplification products were analyzed through the use of electrophoresis in a 1.5% agarose gel conducted at 4V/cm in Tris-borate EDTA buffer. The gels were stained with 0.5 µg/ml ethidium bromide and the PCR products were visualized under 300 nm ultraviolet light. GeneRuler®DNA Ladder Mix (Fermentas GmbH, Germany).served as the molecular weight marker. The identity of each band was determined by visual comparison with a molecular weight ladder. Reactions were deemed positive in the presence of bands of the appropriate size (Figure 1).

Statistical analysis

All data were analyzed by using SPSS (SPSS Inc., Chicago, IL, USA) 12.0 software program for windows. Chi-square test was used to compare

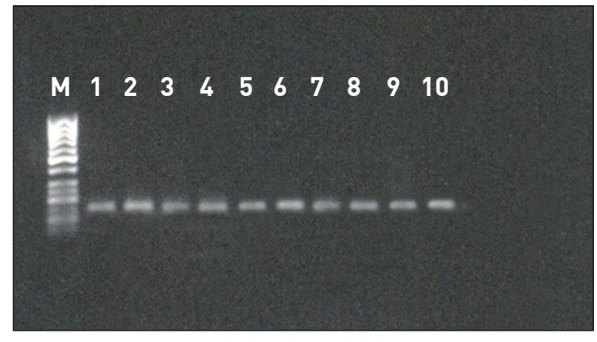

Figure 1. Detection of *E. faecalis* by PCR.
M: Marker DNA (GeneRuler®DNA Ladder Mix (Fermantas))
1-10: *E.faecalis* positive samples

Table 1. PCR primers, with expected amplicon size and thermocycling parameters used in the present study.

Target	Sequence (5' to 3')	Size (bp)	Amplification cycles
Universal 16S rDNA	AGA GTT TGA TCC TGG CTC AG ACG GCT ACC TTG TTA CGA CTT	1505	30 cycles: 94°C 15 s, 54°C 15 s, 72°C 45 s
Enterococcus faecalis	GTT TAT GCC GCA TGG CAT AAG AG CCG TCA GGG GAC GTT CAG	310	36 cycles: 95°C 30 s, 60°C 1 min, 72°C 1 min

the data.

RESULTS

Among 145 children, only 83 (57%) of them had necrotic asymptomatic molar teeth. The presence of *E. faecalis* was evaluated both by culture and PCR methods in these 83 children.

The mean±SD age of the children in deciduous tooth group was 7.56±1.90 years old, while the mean±SD age of the children in permanent tooth group was 10.23±2.10 years old.

The difference in the presence of *E. faecalis* in the root canals between the deciduous (18%) and permanent (26%) tooth groups by culture and PCR methods was statistically significant (P=.03 and .02, respectively). PCR method was found more sensitive than culture method in both deciduous and permanent teeth. However, the difference between culture and PCR methods in both deciduous and permanent tooth groups was not statistically significant (P>.05)(Table 2).

DISCUSSION

The presence of *E. faecalis* in the root canals was detected by culture and PCR methods in the present study. *E. faecalis* was tested because it was reported as therapy-resistant bacteria in the root canals. The success of endodontic treatment depends on several factors, the most important of which is the reduction or elimination of bacterial infection.[11] Therefore, it is important for the clinician to define this bacteria and its growth ability in the endodontic microenvironment.

Culture and molecular methods are used to detect bacterial species in root canal infections. Bacterial culture identifies the predominant species and has played a key role in the association of specific bacteria of endodontic infections.[9,16,21] Molecular techniques, particularly polymerase chain reaction have been used to detect bacteria in endodontic infections. Molecular techniques can detect uncultivable or difficult-to-grow bacteria. Although culture and molecular techniques have been widely used to detect bacteria in endodontic infections,[8,9] few studies have used to investigate the microbiota in deciduous teeth.[11-13]

Earlier studies using culture methods have reported that *E. faecalis* is not normally present or is present in very low numbers in the untreated canals.[22,23] Rocas et al[24] and Fouad et al[25] reported a smaller occurrence (18% and 8%, respectively) of *E. faecalis* using molecular techniques. It was also found similar in the present study both by culture and PCR methods. The presence of *E. faecalis* was also similar in deciduous and permanent teeth groups in the present study.

In the previous reports, the difference between culture and PCR methods was statistically significant. PCR technique was found more sensitive than culture method.[16,26-28] The PCR method was also found more sensitive than culture method in the present study. However, the difference was not statistically significant. There may be two explanations for this result. First; most of the previous reports were performed in secondary infection. The failure of the culture method in those studies should be because of the difficulty during sampling procedure in obturated teeth. In the present study, the sampling procedure was performed in primary infection in both deciduous and permanent teeth. Therefore it can be thought that adequate sample was obtained from each canal for both culture and PCR methods. This was contributed to the similar results with culture and PCR methods. Second; culture methods have some limitations in the detection of especially obligate anaerobic bacteria. Since *E. faecalis* is facultative anaerobic bacteria, both culture and PCR methods showed similar results.

The presence of *E. faecalis* in the root canals of the deciduous teeth of young individuals will add knowledge about the presence of this microorganism in the oral cavity since early stages. This is important for the treatment plan for the clinician. The results of the present study confirm that both culture and PCR methods are sensitive to detect *E. faecalis* in both deciduous and permanent teeth. It should be emphasized

Table 2. Detection of *E. faecalis* by culture and PCR methods.

	Presence of *E. faecalis* by culture N (%)	Presence of *E. faecalis* by PCR N (%)
Deciduous teeth n = 45	8 (18)	10 (22)
Permanent teeth n = 38	10 (26)	12 (32)

that the PCR technique only detected the target species, but did not enumerate the total number of bacteria present in the samples. This points out the importance of the culture method additionally molecular methods.

CONCLUSIONS

The results of the present study confirm that both culture and PCR methods are sensitive to detect *E. faecalis* in root canals.

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